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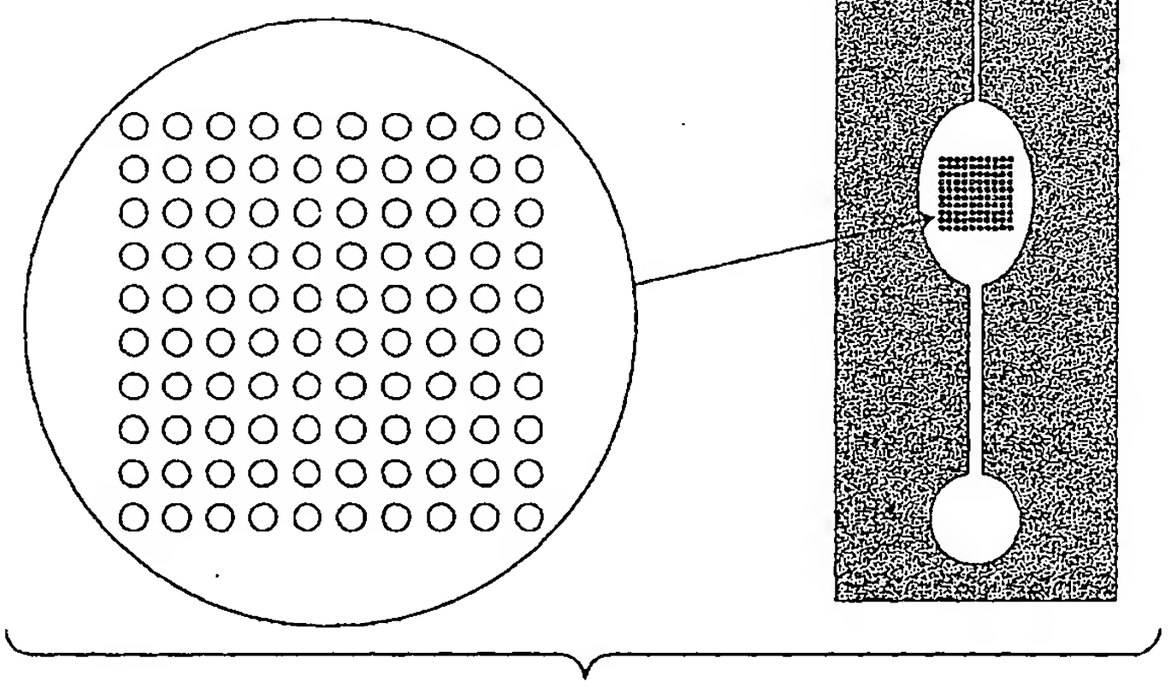

(57) Abstract: The invention provides methods for analyte detection and quantitation using array-based systems.

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ARRAY-BASED ANALYTE DETECTION

Related Applications

This application claims priority to U.S. Provisional Application Serial No. 60/840,088, filed August 25, 2006, and entitled "CHIP-BASED METHODS AND COMPOSITIONS FOR DETECTION OF ANALYTES", the entire contents of which are incorporated by reference herein.

Background of the Invention

Detection of analytes has a number of different applications in a number of fields including the medical and research fields. Prior art methods for detecting analytes generally employ bulk fluorescent measurements and thus are not able to enumerate analytes individually. In addition, prior art flow-based methods generally require sample volumes (or analyte concentrations) in excess of those that are attainable. As a result, there exists a need for methods that detect analytes individually, particularly from rare and/or low volume sample sources.

Summary of the Invention

The invention relates broadly to the detection of analytes using single molecule detection methods and systems. The methods and systems provided herein employ solid substrate chips upon which binding assays, such as but not limited to heterogeneous sandwich assays, are performed in order to detect, and in some instances enumerate, analytes from a variety as well as a plurality of samples.

Thus, in one aspect, the invention provides a method for detecting an analyte in a sample comprising contacting a primary analyte binding partner immobilized on an array with a sample for a time and under conditions sufficient to allow an analyte in the sample to bind specifically to the primary analyte binding partner, contacting an analyte specifically bound to the primary analyte binding partner with a secondary analyte binding partner that is conjugated to a fluorophore, and detecting fluorophores conjugated to a single secondary analyte binding partner and bound to the array, wherein the fluorophores bound to the array are indicative of analytes in the sample. In some embodiments, the analytes are also enumerated.

In another aspect, the invention provides a method for detecting an analyte in a sample comprising contacting a primary analyte binding partner immobilized on an array with a sample for a time and under conditions sufficient to allow an analyte in the sample to bind specifically to the primary analyte binding partner, wherein analytes in the sample are conjugated to fluorophores, and detecting fluorophores that are conjugated to a single analyte and bound to the array, wherein the fluorophores bound to the array are indicative of analytes in the sample. In some embodiments, the analytes are also enumerated.

In some embodiments, the array is comprised of a plurality of different primary analyte binding partners each plurality immobilized on a known region of the array. The plurality of different primary analyte binding partners may be at least 10, 50, or 100 or more different primary analyte binding partners.

In some embodiments, the secondary analyte binding partner is a plurality of different secondary analyte binding partners. The plurality of different secondary analyte binding partners may be at least 10, 50, or 100 or more different secondary analyte binding partners.

In some embodiments, the plurality of secondary analyte binding partners are conjugated to identical fluorophores.

In some embodiments, the analytes are conjugated to identical fluorophores.

In some embodiments, the fluorophores are detected using a multi-pixel charge coupled device (CCD) camera. The multi-pixel CCD camera may have a pixel array of at least 128 x 128 pixels but is not so limited. The multi-pixel CCD camera may have a magnification range of 1-100 but is not so limited. The multi-pixel CCD camera may have an optical resolution of one micron but is not so limited.

In another aspect, the invention provides a chip comprising a configuration comprising an inlet port, an inlet channel, a reaction chamber comprising at least one array of primary analyte binding partners, an outlet channel, and an outlet port.

In some embodiments, the ports, channels and chamber all comprise and are connected by smooth walls. In some embodiments, the total volume of the channels and chamber is less than or equal to 1 microliter. In some embodiments, the ports, channels and chamber are defined by the absence of hydrophobic ink on the chip surface. In some embodiments, the chip is made from low fluorescence fused silica. In some embodiments, the chip is coated with metal and metal oxide. In some embodiments, the

inlet and/or outlet channel has a length of 1-20 mm. In some embodiments, the outlet channel has a width that decreases towards the outlet port. In some embodiments, the inlet and outlet channels have a combined volume that is less than 1-10% of the reaction chamber volume. In some embodiments, the chip further comprises a fiducial. In some embodiments, the reaction chamber comprises at least 2 arrays. Each of the at least 2 arrays may be at least a 10 x 10 array. In some embodiments, the chip comprises a plurality of configurations. The plurality of configurations may be identical to each other. In some embodiments, the array is at least a 10 x 10 array.

These and other embodiments of the invention will be described in greater detail herein.

Each of the limitations of the invention can encompass various embodiments of the invention. It is therefore anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention. This invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

The phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The use of "including", "comprising", or "having", "containing", "involving", and variations thereof herein, is meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

Brief Description of the Figures

FIG. 1 is a schematic representation of a chip having one inlet port, one inlet channel, one reaction chamber having one array, one outlet channel and one outlet port.

FIG. 2 is a schematic of background readings from a 128 x 128 pixel camera image (i.e., illumination spot).

FIG. 3 is a graph showing the readout of immobilized fluorophores using the methods of the invention.

FIG. 4A is a three-dimensional graph showing background signal output.

FIG. 4B is a three-dimensional graph showing fluorescent microsphere signal output.

It is to be understood that the Figures are not required for enablement of the invention.

Detailed Description of the Invention

The invention in its broadest sense relates to the detection of analytes using arrays. The invention detects and optionally enumerates analytes using single analyte sensitivity rather than bulk analyte detection. The invention also requires small sample volumes for analyte detection. Given the multiplexing power of arrays, the methods provided herein are able to detect analytes from nanoliter volumes (e.g., 1-10 nanoliter per array on a chip). Flow-based methods of analyte detection require larger sample volumes.

Single analyte sensitivity, as used herein, refers to the ability to detect and optionally enumerate analytes individually and independent of the presence of other analytes in a sample or on an array. Thus, the methods provided herein do not employ bulk or population measurements and/or averages to determine the presence and/or number of analytes in a sample. The methods of the invention are referred to as non-averaged methods. In the array and pixel context described herein, this means that the presence or absence of analytes bound to the array will be based on a per pixel analysis rather than a total pixel analysis (or alternatively on a per analyte analysis rather than an analysis of the analyte population. These methods therefore do not sum signal from all pixels intended to detect one particular analyte, nor do they average a sum signal over the total number of pixels. Instead these methods measure signal from individual pixels and compare such signal to background (which may be an averaged number). Pixels having signals that are significantly greater than background indicate the presence of an analyte. As used herein, significantly greater means at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, 350%, or 400% greater (or more) than a background pixel signal (which itself may be an averaged number).

The methods of the invention are useful for various applications including but not limited to biomarker discovery, development and validation, diagnostic testing, therapeutic monitoring (e.g., disease-specific protein panels), rare target/sample detection, analysis of knock-down cell lines, gene expression assays, reporter assays, drug candidate screening (primary and secondary), and drug toxicity studies.

The invention contemplates the use of direct and indirect labeling methods for detection of the analytes of interest. Examples of direct and indirect methods are described below.

In one embodiment, the detection method is a heterogeneous sandwich based assay. A heterogeneous assay is one in which the sample to be tested for analyte presence is contacted with the primary analyte binding partner for a time and under conditions sufficient to allow the analyte (if present in the sample) to bind specifically to its partner, following which unbound sample is washed away, and secondary analyte binding partner conjugated to a detectable label is added for a time and under conditions sufficient to allow it to bind specifically to the analyte. Unbound secondary analyte binding partners are then washed away and buffer is added. The array is then analyzed for the presence and location of detectable label. The presence of the detectable label indicates that an analyte is present in the sample. The location of the detectable label indicates which analyte is present in the sample.

In another embodiment, the detection method involves first detectably labeling any analytes in the sample and then contacting the labeled analytes to the bound primary analyte binding partner. No secondary analyte binding partner is used in this embodiment. This approach preferably is used where all the analytes being detected are of the same nature, in order to ensure equivalent labeling in the first step. As an example, this approach could be used to detect different RNA species by first end-labeling all RNA in the sample and then contacting the labeled sample to the chip array having, for example, nucleic acid probes as the primary analyte binding partner.

In yet another embodiment, the detection method involves first specifically capturing analytes on the array and then detectably labeling the captured analytes (e.g., using primer extension reactions, etc.).

The detection methods of the invention are able to provide a high degree of multiplexing. As used herein, multiplexing is the ability to detect two or more different analytes at a given time in a single reaction run. The degree of multiplexing will depend on the particular application and the number of analytes to be detected. The detection methods are able to detect tens, or even hundreds of analytes in a single reaction run. This is accomplished by using an array. An array, as used herein, means an arrangement of regions positioned on a solid substrate such as for example a chip, wherein one or more of the regions is capable of binding to an analyte. The array may be of any shape,

although square arrays are most common. An example of a square array is a 10 x 10 grid array. Such an array can detect up to 100 different analytes.

The chips provided by the invention may comprise one or more arrays, with each array optionally being used to analyze a separate and independent sample. Some or all of the arrays on a single chip may be identical. If they are all identical, the chip may be used to analyze several different samples in parallel for the presence of the same analytes. As an example, the samples may be from different subjects (e.g., different human patients) or from samples harvested from the same source at different times (e.g., during a time course analysis). A single chip may contain any number of arrays, in any possible configuration. In some embodiments, samples may be transferred from 96 well plates (which have an 8 x 12 grid pattern), and therefore the chips may contain 8 or 12 duplicate arrays (and configurations comprising inlet and outlet ports and channels, as described in greater detail herein). In these embodiments, the inlet ports may be spaced sufficiently apart from each other to provide compatibility with multi-channel pipettes and sample handling robots. A 9mm spacing may be suitable in some instances.

As mentioned, the chips also contain one inlet and one outlet port. In some instances the chips contain one inlet and one outlet port per array and thus each array can be loaded separately from the others. Once sample is added and unbound elements are washed out of the chip, the chip may be configured (together with its loading device) so that all the arrays may be loaded with the same solutions including wash solutions and secondary analyte binding partner solutions.

If the analytes are detected using fluorescent detection, then chips fabricated from very low fluorescence substrate (such as fused silica) or chips coated with metal to mask fluorescence of the chip substrate are suitable for obtaining high signal to noise (i.e., background) ratio during analyte detection. If a metal coating is used, then an additional metal oxide layer may be applied to the chip. The metal oxide layer typically could be greater than 100 microns in depth (e.g., up to 150, 200, 250, 300, 350, 400, 450, 500 or more microns), although the metal oxide layer could also be less than 100 microns in depth (e.g., at least 50, 60, 70, 80 or 90 microns). The metal oxide layer is useful to reduce reflection of the excitation source back into the detector, prevent the metal from contacting and denaturing the sample, and/or prevent the metal from quenching the fluorescence of the probes.

The arrays, channels and ports present in the chip may be etched into the chip using acid or laser etching. Alternatively they may be printed thereon using, for example, hydrophobic ink and an ink jet printer. In the latter embodiment, absence of ink defines the channel, port, and reaction chamber. A suitable ink is one that consists of fluorinated hydrocarbons (e.g. Teflon) to minimize interactions between the sample and the ink. The chips typically contain a cover slip in order to reduce evaporation of the sample and/or solutions.

FIG. 1 is an illustration of a chip with a single 10 x 10 array. The sample and other solutions enter the chip through the inlet port, and travel through an inlet channel which connects the inlet port to the reaction chamber where the array(s) are located. This channel should be narrow enough to allow spontaneous filling by capillary action of all grids in the given array. An outlet channel flows from the reaction chamber to the outlet port. Preferably, the walls of the inlet port are smooth to prevent shaving of plastic off pipette tips. The port volume would be large enough to accommodate the entire dispensed sample (e.g., 1 to 10 microliters). It is to be understood that where the ports, channels and chambers are made using hydrophobic ink, the sides of the ports, channels and chambers will be hydrophobic while the floor and ceiling of the ports, channels and chambers will be hydrophilic.

The reaction chamber is the area on the chip in which the array(s) is located and thus where the binding reaction occurs. Accordingly, it can also be regarded as the binding or hybridization chamber. The volume of the chamber (and hence the volume of sample that interacts with the array) is minimally defined by its shape and the chamber depth.

The outlet channel allows air to escape during filling of the chamber, and to provide a through path for washing the chamber. The outlet channel may have a constant width along its length, or it may become narrower or wider as it approaches the outlet port. Such changes in width may be employed to stop the sample from reaching the edge of the chip by locally increasing the hydrophobicity of the channel, and thereby prevent siphoning or evaporation.

The inlet and outlet channels preferably have negligible volumes compared to the reaction chamber, and particularly in the case of the inlet channel they may be intentionally long to prevent diffusion of analytes from the inlet port to the chamber. In some embodiments, the inlet channel may be a serpentine channel in order to increase

channel length without increasing chip area. Alternatively or additionally, the outlet channel may have a narrower width than the first channel and/or the outlet port may be lined with a hydrophilic substance (such as silane coupling agents with hydrophilic groups (to treat the glass surface), hydrophilic cross-linkable polymer coatings, plasma pre-treatment of glass slides, etc.) in order to draw sample or solution towards it.

These chip configurations provide one or more advantages. For example, accurate pipetting of the sample is not required as long as the dispensed volume is greater than that of the chamber plus channel volumes. The smooth channel geometry prevents "dead volumes" or trapped air pockets during filling and allows the liquid meniscus to advance evenly from the inlet port to the outlet port without trapping small air bubbles. In addition, after spontaneous filling, there will be no further hydrodynamic flow caused by siphoning or evaporation. Although excess sample may evaporate from the inlet port, this will occur without pulling fluid back out of the chamber. The inlet flow rate allows uniform distribution of the sample within the chamber before a significant number of binding events can occur. Flow rates may vary but preferably are on the order of 1 µl/1-10 seconds. The chamber is also large enough to keep the ink walls far enough away from the array to prevent fluorescence of the ink from being detected.

The chamber area is determined by the array and array spot size, which is in turn determined by the surface chemistry and robotics used to apply such chemistry. Typical spot sizes will range from about 50 microns to about 500 microns in diameter, with a similar distance between each spot. Therefore, as an example, a 10 x 10 array of spots will range in size from 1 -10 mm on a single side. Additional clearance may be required around the array to facilitate spotting of the array. The depth of the reaction chamber will be determined by the method used to create the channels and chambers, but will typically range from up to about 10 microns for etched chips to about 100 microns or more for ink printed chips. The chamber volume therefore could range from about 5-10 nanoliters to about 5-10 microliters. These geometries are intended to be representative only and one of ordinary skill in the art will be able to design a chip geometry suitable for a given application.

As used herein, a smooth channel geometry refers to geometry that excludes straight-edged corners, channels with dead ends, or pockets that trap air. Smooth channel geometry may include inter alia rounded corners. An appropriate geometry is

one that provides even and continuous filling of the channels and chamber by capillary action alone. Ideally, the force on the sample liquid is higher in the narrow channels and lower in the wider chamber, and thus the narrow channels fill rapidly while for example the chamber fills more slowly. The geometry of the chip should also avoid any sudden changes in channel width (i.e., continuous gradual change in channel width is preferred to abrupt step wise changes). In one embodiment, the inlet channel walls gradually widen into the chamber, run parallel around the array area and gradually narrow to the outlet channel.

The chips may be manufactured in a number of ways. Generally the chips are cut to size, ground smooth, and then etched or printed to form the ports, channels and/or arrays. If hydrophobic ink is used, it is allowed to dry before the appropriate surface chemistry is added to the array(s). The surface chemistry may be applied to the entire surface of the chip for convenience since it will not bind (or will be easily washed off) the printed regions on the chip. The array(s) of primary (capture) analyte binding partners may be applied to each chamber using, for example, arraying robotics or other automated processes known in the art. (See, for example, Thompson et al. Trends Microbiol. 2001, 9(4):154-6; Maier et al. J Biotechnol. 1994, 35 (2-3):191-203, US 5,807,522, US 6,979,425, US 7,025,933.) Once the surface chemistry is dried, excess binding partners are washed away. If a cover slip is used, it is situated over the channels and chambers but not the inlet and outlet ports. The cover slip may be bonded into place using UV curable epoxy. Other fabrication processes can be envisioned and employed.

The binding steps of the sandwich assay can be performed in any environment that provides conditions suitable for specific and efficient binding of the analyte to its binding partners (and vice versa). These steps can be performed manually or by using an automated process and/or device. Preferably, the method is performed by placing the chip in a device with temperature, humidity, and timing controls. Even more preferably, the device has fluid controls also. Commercially available hybridization ovens can be used at least in embodiments in which the analyte is a nucleic acid. (See, for example, Ventana, Applied Biosystems, Agilent, Affymetrix, Thermo Scientific, Cole Palmer, and SciGene hybridization ovens.)

The binding or hybridization device may have the capability of asynchronously (or independently) processing chips. The device may be programmed to maintain nucleic acid analytes at an elevated temperature until the sample is uniformly distributed

throughout the reaction chamber. Once the sample is uniformly distributed, the temperature is lowered to allow binding of analytes to their binding partners to occur.

The device may also be able to control or modulate, as the case may be, pH within the reaction chamber. pH modulation can be accomplished by buffer addition and/or photo-chemistry. Low pH can partially denature antibody binding sites. As a result, pH control is useful in ensuring uniform distribution of samples when antibodies or antibody fragments are used as the primary analyte binding partners. Once sample is uniformly distributed, the pH is raised in order to allow binding to occur.

The device may also be able to block the outlet, and optionally the inlet, port during binding steps in order to prevent flow of sample and/or other solutions out of the chip. The device may unblock the port(s) and perform a wash of the chamber(s) to remove excess sample and unbound analytes. The device may prompt an operator to add sample, wash buffer, or secondary analyte binding partner solution, or it may add these reagents automatically.

The chips are then analyzed for bound analytes. Importantly, the chips are analyzed in a manner that allows detection of individual analytes rather than an estimate of total analytes using bulk measurement techniques. Detection is performed by illuminating each spot in an array with a uniform field of light close to the optimum excitation wavelength of the fluorophore (or other detectable label) bound to the secondary analyte binding partner, in the case of a sandwich assay. The entire spot is imaged using an electron-multiplying charge coupled device (CCD) camera (EMCCD). The EMCCD chip is cooled to about -50°C to make thermal noise from each pixel negligible compared to the number of photo-electrons produced during the exposure time. An EMCCD amplifies the number of photo-electrons to higher than the readout noise of the camera, thereby allowing the camera to be used as a photon-counting device.

It is to be understood that, although the description of the invention recited herein frequently refers to fluorophores as the detectable labels of the method, this is done for the sake of convenience and that the invention broadly contemplates the use of other detectable labels also. Examples of other detectable labels are provided below.

In one instance, the detection system may be single color (e.g., red) detection system. It may use one or more high power light emitting diodes (LEDs) or a laser to excite the fluorophore bound directly or indirectly to the analyte. A coherent or diffraction limited light source may be used but is not essential. Various lens and filter

configurations are possible. For example, a three color polychroic filter or a dichroic mirror may be used to reflect a blue excitation wavelength and transmit the longer fluorescence wavelengths. Additionally, the camera objective comprises a temperature control that maintains a low temperature during analysis of the chip. The objective is also preferably equipped with an oil or water immersion lens, a high numerical aperture (NA) for brightness, and bottom illumination for alignment.

The camera images spots on the array individually. As used herein, a spot on an array refers to a region that has bound to it one particular primary analyte binding partner. For example, a 10 x 10 grid will have 100 spots, and each of these spots will be analyzed separately. Importantly each of these spots is analyzed using high resolution (i.e., high pixel) images from the camera rather than through bulk (e.g., fluorescence) measurement of the spot as a whole.

The camera generates an image for each array spot. The image is set to be equal or slightly larger than the diameter of one array spot (typically 50 to 500 microns in diameter). Beam profiling optics are used to make the illumination intensity over the image as uniform as possible.

The image will consist of a plurality of pixels. The greater the number of pixels, the more sensitive the camera, and thus the greater the ability to detect single analytes bound to the array spot. The ability to dissect the array spot (according to pixels in the image) allows counting of single analytes rather than simply measurement of bulk signal (e.g., fluorescence) from the entire image. Thus, analyte counting is achieved by counting individual analytes (and in some instance individual fluorophores) within each image. This is single molecule detection of each bound analyte instead of the conventional "bulk" readings of the prior art.

The dynamic response will become noticeably non-linear as the probability that two or more analytes (and in some instances two or more fluorophores) occur in a single pixel becomes significant. Increasing the number of pixels in the image increases the dynamic range of the assay. For example, a 128 x 128 pixel image can yield a dynamic range up to 10^4 analytes. A camera with 512 x 512 or even 1024 x 1024 pixels may therefore be preferable in some instances.

The pixel size is chosen in conjunction with the magnification to match the optical resolution of the system. For example, the size of the pixel (on a side) should be equal to, or slightly less than, the optical resolution of the system. The pixel dimensions

therefore should be chosen to be about the same size or slightly larger than the image created by a single analyte (and in some instances fluorophore).

In some instances, the optical resolution of the system is on the order of one micron. If there are insufficient pixels on the camera to map one pixel per micron, then multiple exposures could be taken with slightly different chip positions to resolve closely spaced fluorophores.

FIG. 2 is a schematic of background readings from a 128 x 128 pixel image. Each circle represents signal superimposed on the pixel array. At the bottom of the image, two signals are present in one pixel. This is representative of the multiple analyte (or fluorophore) per pixel instance discussed above. FIG. 3 shows the dynamic range that can be achieved using the CCD camera and chip array of the invention. The data shown in FIG. 3 are from a simulation that counts fluorophores immobilized on a chip similar to that of the invention. The fluorophores are counted using a 128 x 128 pixel image and CCD camera. The Figure shows a relatively linear detection range through about 4 logs of magnitude, and further detection potential up to about 30000 fluorophores before saturation of the system.

Alternatively, the method could be performed using a single pixel camera that is scanned across the array to derive a comparable image. It is preferable however to use a camera having as many pixels are possible for convenience as will be clear from the disclosure herein.

The array based methods can employ the same excitation devices as used in flow based methods, including lasers, without a need for increased power output. This is so even though the spot being imaged is greater (and therefore the power per unit area is lower) than that of flow based methods. The excitation intensity per unit area on the array is typically about 400 times lower than that of flow based methods (e.g., from 1.2 milliwatt per squared micron using a flow based system to 0.003 milliwatt per squared micron using the array based system). In order to overcome this reduction in power, the array based methods increase exposure times as compared to flow based methods. The exposure time using an array based method can typically be 5000-10000 times longer than the exposure time for a flow based method (e.g., from about 100 microseconds using a flow based system to 0.5 to 1 second using the array based system). In addition, the array based methods have no requirement for pre-bleaching, and a 2-fold increase in

optical efficiency. These parameters result in an estimated 50-fold overall increase in peak intensity using the array based system.

FIG. 4A and 4B provide a comparison of signal output of background (left, 4A) and fluorescent micropheres (right, 4B) using the methods of the invention. The Figures are images of a TetraSpeck™ Fluorescent Microsphere Standard slide, illuminated with a high power red LED running at 1 Ampere forward current and taken with an exposure time of 1 second. An oil immersion objective lens was used, with a focal length of 2 mm and a NA of 1.40. A dichroic filter was used to reflect the excitation light onto the slide but transmit the fluorescent light through to the camera. No attempt was made to provide uniform illumination across the field of view. The focus of the system was adjusted to maximize signal height (and minimize peak width) from the individual spheres. The Figures were obtained from a 128 x 128 pixel EMCCD in photon-counting mode. Each represents a graph of the camera output (in the Z axis) for each pixel (as plotted on the X and Y axes). There are 16384 data points in each Figure. The Figures are representative of the images generated by the camera.

FIG. 4A is a region of the slide that does not contain microspheres and represents the noise or background of the system. The noise is primarily stochastic noise from the amplification stage of the camera. The CCD element itself was cooled to -50 °C so dark noise from the pixels is negligible, and the camera gain was increased to ensure that readout noise from the camera's A/D stage is also negligible. FIG. 4B is a region of the slide containing 0.1 micron diameter, red fluorescent microspheres. The optical resolution of the system had previously been determined to be between 1 and 2 microns, so the spheres are essentially "point sources" as a single fluorescent dye molecule would be. Three spheres are visible in the field of view and are seen as spikes in the signal from those pixels that correspond to the location of the spheres. The spike in the center is probably larger than the others due to higher illumination near the center of the field of view.

Data from the camera images may be processed in a number of ways. The following exemplary analysis involves a two step process that accounts for optical or electrical noise from the system as well as background noise attributable to other known or unknown sources. The desired output of analyte detection analysis is to count the number of analytes (or optionally fluorophores in some instances) bound to the chip (e.g., via the direct and indirect labeling techniques described herein). In the absence of

any non-specific binding or cross-talk with other analyte species, and in instances in which each analyte is represented by only one fluorophore, the number of fluorophores counted will equal the number of analytes bound to the chip. In one approach, the number of pixels having signals significantly higher (e.g., at least 100%, 200%, 300%, or 400% or higher) than the background of the detection system (e.g., when no fluorophores are present) represent the number of analytes. For example, background can be measured by taking an image of a region of the chip where no fluorophores can bind (e.g., from a dedicated chamber that has never been exposed to the labeled binding partner), and summing the signal from that region. The mean and standard deviation of the signal from all pixels from that background region is then calculated and the threshold is set depending on the desired false negative rate (e.g., mean plus 3 standard deviations). The number of pixels with signals that significantly exceed this threshold is then counted and reported for each spot.

In some embodiments, the assay will also comprise running one or more negative controls which are identical in all aspects to test samples, except that the negative control would contain no target analyte. Typically, some bound fluorescence will be detected due to non-specific binding, cross-reactivity with background components, and imperfect washing of the chamber. By running a large number of negative controls (e.g., 10 or 20) the average and standard deviation of this type of background can be determined. The limit of detection of the assay may be set at the sample concentration that produces a number of fluorescent pixels significantly higher than the average number of background fluorescent pixels (e.g., 2, 3, 4, 5, 10, 20, 50, 100 fold or more than the background pixel number).

FIGs. 4A and 4B demonstrate the sensitivity of the single molecule detection approach over a bulk (e.g., fluorescence) detection approach as follows. A bulk fluorescence reader integrates all the signal emitted from each spot in an array. As a result, the output from such a reader is equivalent to adding the counts from the individual pixels and arriving at a "brightness" sum. In the case of FIG. 4A, this sum total would be on the order of just over 24,000,000 counts, with an average of about 1480 counts per pixel. The reading from the pixel corresponding to the largest peak in FIG. 4B is 6500 counts, or approximately 5000 counts above background average. The signal to noise ratio for one microsphere using the single molecule approach is therefore 5000/1480 or 379%. If the data from FIG. 4B had been analyzed by a fluorescent reader

using a bulk fluorescent approach, then the one microsphere would increase the total fluorescent reading to 24,005,000 or 0.02% above the background average. This increase is effectively undetectable, given that most if not all bulk readers would have reproducibility variations higher than that. For example, in this analysis repeated readings of background noise gave a CV of 0.06%.

Each reading consists of activating the light source for a precise time and capturing one exposure over the same interval. The irradiance of the excitation source and the exposure time are designed so that all the fluorophores will photo-bleach during the exposure. This extracts as many photons as possible from the fluorophores, and makes the number of photons emitted from each fluorophore as similar as possible. The light source is only turned on during data acquisition to prevent photo-bleaching of the fluorophores during alignment rather than exposure.

Previous experiments with single molecule detection in a flowing stream have shown that TAMRA dyes saturate under a laser intensity of 1–2 mW per square micron, and exposure times of 0.1 mS. A 100 micron diameter spot has an area of 7584 square microns, so a corresponding increase in exposure time (to 758mS or ¾ of a second) would preserve a similar number of excitation photons per square micron. Conditions for photobleaching are heavily dependent on the chosen fluorophore, attachment chemistry and assay conditions, but such conditions can be readily found by routine experimentation.

Preferably, the array spot fills the entire active area of the CCD chip. This is accomplished by increasing the magnification factor of the camera lens as necessary. It results in the maximum number of pixels to image the array spot. In some instances, the maximum number of fluorophores that can be counted (even though there may be many more in view) is equal to the number of pixels in the image.

As noted previously, it is desirable to choose the optical magnification so that the size of each pixel in the camera is equal to, or smaller, than the optical resolution of the system. Pixel sizes can range from a few microns to 25 microns on a side, and the optical resolution can be less than 1-2 microns, depending on the laser wavelength and optics employed. Typical magnifications are therefore in the range 2 to 50.

The concentration of the primary analyte binding partners on the surface of the chip should be chosen to match the dynamic range of the assay and the number of pixels of the camera. For accurate quantitation at high analyte concentrations, the number of

primary analyte binding partners should be in excess of the largest expected analyte concentration. Due to saturation effects, the greatest number of analytes that can be accurately counted is less than the number of pixels of the camera.

Notwithstanding the caveats set forth herein, in instances in which the fluorescence intensity from each fluorophore is sufficiently uniform and consistent, such intensity values can be used to count more than one analyte per pixel. This would increase the dynamic range beyond the number of pixels in the image.

In certain embodiments where scattered light from one spot causes photo-bleaching into adjacent spots, it may be preferable to space the array spots from each other in order to avoid or eliminate such photo-bleaching.

An anti-bleaching agent could be added to the buffer to increase the number of photons obtained from each fluorophore. Preventing long term photo-bleaching will increase the signal from each fluorophore.

In some embodiments, a long wavelength (e.g., an infrared wavelength) is used to illuminate the array spot. This is useful in aligning the spot to the CCD camera without photo-bleaching the fluorophores. Alignment can also be facilitated by detecting fluorescence from the ink and using such fluorescence as an alignment guide. Alternatively or additionally, a fiducial can be etched into or printed onto the slide in order to locate a starting point of analysis for each array.

The chip may be mounted in a re-usable cartridge prior to placement in the detection system. This cartridge could provide the following functionality: (a) reproducible alignment, (b) a well above the chip to hold the index-matching liquid (oil or water), and/or (c) a lid to keep the chip in the dark until it is in place in the detection system.

The invention provides high throughput analysis of samples. The lower limit of detection is about 0.1 pg/ml or 2 fM although the method can theoretically yield a limit of detection of about 200 aM, assuming a CV of about 10% which requires a minimum of 100 detected events. This number of analytes in a 1 microliter volume corresponds to 0.17 fM (or roughly 200 aM). The total sample consumption per chamber is about 1 microliter. The dynamic range of analyte quantitation is about 4 logs. The level of reproducibility (i.e., the coefficient of variance) is about 10%. Using a single 10 x 10 array, it is expected that 100 assays can be performed on a given sample. Analysis

including detection and readout is expected to take about 4 hours to analyze each sample in a 96 well plate using a 10 x 10 spot array.

The analytes can be organic or inorganic in nature, and in important embodiments, they include pathogens such as bacteria, viruses, fungi, parasites, mycobacteria, as well as proteins, peptides, lipids, carbohydrates, toxins such as microbial toxins, and nucleic acids derived from such pathogens or from other sources. The nucleic acid may be naturally or non-naturally occurring DNA or RNA, such as genomic DNA, mitochondrial DNA, mRNA, miRNA or cDNA. Peptides and protein analytes can be detected, for example, using antibodies or antibody fragments. Nucleic acid analytes can be detected, for example, using oligonucleotides. In a particular example, modified oligonucleotides comprising locked nucleic acid (LNA) residues or regions or 2'-O-methyl modified bases can be used for short nucleic acid analytes such as but not limited to miRNA.

The assay can be used to detect and quantify analytes directly in biological samples such as whole blood, blood serum, and cell lysates. Whole cells could also be analyzed by replacing the capture probe array with an immobilized cell array. The cells could then be interrogated by washing out the original medium and replacing it with reporter probes that bind to specific cell structures, or molecules within the cell (e.g. FISH). A combination of the above two approaches could be used to analyze cell contents by performing in-situ lysis of immobilized cells. In this embodiment, the chamber contains a capture area for whole cells and an array for intracellular targets. After washing, the cells would be lysed in the chamber followed by an incubation period to allow the cell contents (e.g., proteins, DNA, mRNA, miRNA, etc.) to bind to the array. The cell contents that are not of interest can be washed out and replaced with reporter probes for the analytes. Reading the array would proceed as described herein.

Harvest and isolation of nucleic acids are routinely performed in the art and suitable methods can be found in standard molecular biology textbooks. (See, for example, Maniatis' Handbook of Molecular Biology.)

The invention is not limited to the type of analyte that can be detected provided that the analyte has a binding partner. An analyte binding partner is a molecule that preferably binds to the analyte rather than to other molecules or compounds. To preferably bind the analyte means to bind to the analyte with greater affinity than to other molecules or compounds. Thus for example the analyte binding partner may bind to the

analyte with an affinity that is 5-fold, 10-fold, 50-fold, 100-fold, 1000-fold, or greater than its affinity for any other molecule.

The analyte binding partner may be an antibody, an antibody fragment that binds the analyte (e.g., Fab, F(ab)2, Fv, etc.), a peptide that binds the analyte, a nucleic acid that binds to the analyte via hybridization and thus complementarity of sequence, a nucleic acid that binds to the analyte via structural affinity (e.g., an aptamers), a carbohydrate, a lipid, a chemical compound, or any other molecule, or any combination of the afore-mentioned molecules.

The primary analyte binding partner (i.e., capture probe) is bound (or fixed) to a solid substrate (i.e., the chip). The secondary analyte binding partner (i.e., reporter probe) is free-flowing until it is bound to the analyte in its captured form. Preferably, the capture and reporter probes bind to separate regions on an analyte regardless of whether those regions are identical in terms of sequence or structure. In other words, binding of either the capture or reporter probes should not effectively compete with or interfere with the binding of the other to the analyte. Preferably, the reporter probes are detectably labeled.

In most instances involving a sandwich assay, the primary analyte binding partner is not detectable while the secondary analyte binding partner is detectable. The secondary analyte binding partner is generally detectable or capable of being detected. For example, the secondary analyte binding partner may be detectable because it is itself conjugated to a detectable label such as a fluorophore. The method envisions labeling different secondary analyte binding partners with the same detectable label. In this way, only one excitation source and detector are necessary to detect a variety of analytes.

In instances involving directly detectably labeled analytes, the analytes are labeled with a detectable label and no secondary analyte binding partner is employed. The method envisions labeling different analytes with the same detectable label for the same reasons as stated above.

Examples of suitable detectable labels include fluorophores, microspheres loaded to conjugated to fluorophores, microspheres that are inherently fluorescent (e.g., polystyrene microspheres), quantum dots, particles that scatter light (e.g., gold particles), chemiluminescent molecules, and the like. Further examples of detectable labels are provided below.

The analyte binding partners can be of any nature including but not limited to nucleic acid (e.g., aptamers), peptide, carbohydrate, lipid, and the like. A common form of binding partner is an antibody or an antigen-binding antibody fragment, particularly for non-nucleic acid analytes. Antibodies include IgG, IgA, IgM, IgE, IgD as well as antibody variants such as single chain antibodies. Suitable antibody fragments contain an antigen-binding site and thus include but are not limited to Fab and $F(ab)_2$ fragments. A nucleic acid based binding partner such as an oligonucleotide can be used to recognize and bind DNA or RNA based analytes. The nucleic acid based binding partner can be DNA, RNA, LNA or PNA, although it is not so limited. It can also be a combination of one or more of these elements and/or can comprise other nucleic acid mimics.

The invention can be applied to the detection and optionally identification and/or quantification of any analyte, including rare analytes which would otherwise be costly to detect.

One example of an analyte to be detected according to the invention is a genetically modified organisms (GMO) (or byproduct thereof) in a food source. Examples of GMOs and food sources include plants that are modified to include genes that confer resistances to disease, insects, or weed killers, and plants and animals that include genes to increase their yield of human and livestock food stuff (e.g., milk, meat, corn kernels, etc.). The analyte to be detected from a GMO may be a nucleic acid corresponding to one of these phenotypes. It will be appreciated that a single GMO may comprise more than one modified gene and the method may be used to detect one or more of those genes.

Another example of an analyte to be detected according to the invention is a biohazardous or biowarfare agent. These agents can be biological or chemical in nature. Biological biowarfare agents can be classified broadly as pathogens (including spores thereof) or toxins. As used herein, a pathogen (including a spore thereof) is an agent capable of entering a subject such as a human and infecting that subject. Examples of pathogens include infectious agents such bacteria, viruses, fungi, parasites, mycobacteria and the like. Prions may also be considered pathogens to the extent they are thought to be the transmitting agent for CJD and like diseases. As used herein, a toxin is a pathogen-derived agent that causes disease and often death in a subject without also causing an infection. It derives from pathogens and so may be harvested from such pathogens. Alternatively, it may be synthesized apart from pathogen sources.

Biologicals may be weaponized (i.e., aerosolized) for maximum spread. Examples of biowarfare agents include those listed and categorized by the CDC.

CDC Category A agents include Bacillus anthracis (otherwise known as anthrax), Clostridium botulinum and its toxin (causative agent for botulism), Yersinia pestis (causative agent for the plague), variola major (causative agent for small pox), Francisella tularensis (causative agent for tularemia), and viral hemorrhagic fever causing agents such as filoviruses Ebola and Marburg and arenaviruses such as Lassa , Machupo and Junin.

CDC Category B agents include Brucellosis (Brucella species), epsilon toxin of Clostridium perfringens, food safety threats such as Salmonella species, E. coli and Shigella, Glanders (Burkholderia mallei), Melioidosis (Burkholderia pseudomallei), Psittacosis (Chlamydia psittaci), Q fever (Coxiella burnetii), ricin toxin (from Ricinus communis – castor beans), Staphylococcal enterotoxin B, Typhus fever (Rickettsia prowazekii), viral encephalitis (alphaviruses, e.g., Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis), and water safety threats such as e.g., Vibrio cholerae, Cryptosporidium parvum.

CDC Category C agents include emerging infectious diseases such as Nipah virus and hantavirus.

Other pathogens that can be detected using the methods of the invention include N. gonorrhea, H. pylori, Staphylococcus spp., Streptococcus spp. such as Streptococcus pneumoniae, Syphilis; viruses such as SARS virus, Hepatitis A, B and C viruses, Herpes virus, HIV, West Nile virus, Influenza A virus, poliovirus, rhinovirus; and parasites such as Giardia.

Examples of toxins include abrin, ricin and strychnine. Further examples of toxins include toxins produced by Corynebacterium diphtheriae (diphtheria), Bordetella pertussis (whooping cough), Vibrio cholerae (cholera), Bacillus anthracis (anthrax), Clostridium botulinum (botulism), Clostridium tetani (tetanus), and enterohemorrhagic Escherichia coli (bloody diarrhea and hemolytic uremic syndrome), Staphylococcus aureus alpha toxin, Shiga toxin (ST), cytotoxic necrotizing factor type 1 (CNF1), E. coli heat-stable toxin (ST), botulinum , tetanus neurotoxins, S. aureus toxic shock syndrome toxin (TSST), Aeromonas hydrophila aerolysin, Clostridium perfringens perfringolysin O, E. coli hemolysin, Listeria monocytogenes listeriolysin O, Streptococcus pneumoniae pneumolysin, Streptococcus pyogenes streptolysine O, Pseudomonas aeruginosa

exotoxin A, E. coli DNF, E. coli LT, E.coli CLDT, E. coli EAST, Bacillus anthracis edema factor, Bordetella pertussis dermonecrotic toxin, Clostridium botulinum C2 toxin, C. botulinum C3 toxin, Clostridium difficile toxin A, and C. difficile toxin B.

Examples of chemicals that can be detected include arsenic, arsine, benzene, blister agents/vesicants, blood agents, bromine, borombenzylcyanide, chlorine, choking/lung/pulmonary agents, cyanide, distilled mustard, fentanyls and other opioids, mercury, mustard gas, nerve agents, nitrogen mustard, organic solvents, paraquat, phosgene, phosphine, sarin, sesqui mustard, stibine, sulfur mustard, warfarin, tabun, and the like.

The methods can detect a plurality of analytes using a plurality of binding partners. A plurality as used herein is more than one and can be at least 3, at least 4, at least 5, at least 10, at least 25, at least 50, at least 75, at least 100, at least 200, at least 500, at least 1000, at least 5000, at least 10,000, at least 20,000, at least 50,000, or more.

The sample to be tested for analyte presence and/or amount can be derived from virtually any source and will depend primarily on the analyte being detected. The sample may be a biological sample from a subject such as a bodily fluid or tissue. The term tissue as used herein refers to both localized and disseminated cell populations including, but not limited, to brain, heart, breast, colon, bladder, uterus, prostate, stomach, testis, ovary, pancreas, pituitary gland, adrenal gland, thyroid gland, salivary gland, mammary gland, kidney, liver, intestine, spleen, thymus, bone marrow, trachea and lung. Biological fluids include saliva, sperm, serum, plasma, blood, lymph and urine, but are not so limited. Both invasive and non-invasive techniques can be used to obtain such samples and these are known to those of ordinary skill in the art.

Accordingly, the methods and devices provided herein can be used to detect biological analytes such as those found naturally in an individual (e.g., a human) or those found only in association with a disease or condition, including those used to identify risk of a disease or condition (e.g., CRP). In these respects, the analyte may be a factor routinely tested in a laboratory setting (e.g., cholesterol, lipids, pregnancy determining factors, and the like). It may also be a tumor cell or a tumor antigen that is shed from a tumor and is present in the blood stream. Those of ordinary skill in the art will understand and appreciate the breadth of analytes that can be assayed according to the invention.

Alternatively, the sample may be an environmental sample such as an air sample or a water sample. In this latter embodiment, the sample may be checked for, for example, chemical or biological warfare agents such as those recited herein. If the sample is an air sample, it will generally require dissolution in a liquid base such as a buffered solution. This is usually also the case with solid samples.

The analyte being detected can dictate whether the sample needs to be further manipulated prior to analysis. In some embodiments, it may be necessary to disrupt larger analytes such as pathogens prior to contact with the probe. Disruption can be mechanical, including acoustic disruption (e.g., ultrasound based disruption), and may be carried out to varying degrees. For example, a sample may be disrupted to the point of rupturing cell walls and/or cell membranes and releasing cell wall fragments, intracellular organelles, proteins, lipids, and/or genomic DNA, all of which may be analytes.

Depending on the expected concentration of the analyte being detected, the sample may be diluted or concentrated prior to analysis. Dilution will generally involve mixing of the sample with a larger volume of solution. Concentration can be accomplished in a number of ways known in the art including but not limited to centrifugation, filtering, and the like. Concentration may also be accomplished using flow directed concentration methods.

In some embodiments, the invention embraces nucleic acid derivatives as binding partners or probes. As used herein, a "nucleic acid derivative" is a non-naturally occurring nucleic acid or a unit thereof. Nucleic acid derivatives may contain non-naturally occurring elements such as non-naturally occurring nucleotides and non-naturally occurring backbone linkages. These include substituted purines and pyrimidines such as C-5 propyne modified bases, 5-methylcytosine, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, 2-thiouracil and pseudoisocytosine. Other such modifications are well known to those of skill in the art.

The nucleic acid derivatives may also encompass substitutions or modifications, such as in the bases and/or sugars. For example, they include nucleic acids having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus, modified nucleic acids may include a 2'-O-alkylated ribose group. In addition, modified nucleic acids may include sugars such as arabinose instead of ribose.

The nucleic acids may be heterogeneous in backbone composition thereby containing any possible combination of nucleic acid units linked together such as peptide nucleic acids (which have amino acid linkages with nucleic acid bases, and which are discussed in greater detail herein). In some embodiments, the nucleic acids are homogeneous in backbone composition.

Binding partners or probes comprising nucleic acid components can be stabilized in part by the use of backbone modifications. The invention intends to embrace, in addition to the peptide and locked nucleic acids discussed herein, the use of the other backbone modifications such as but not limited to phosphorothioate linkages, phosphodiester modified nucleic acids, combinations of phosphodiester and phosphorothioate nucleic acid, methylphosphonate, alkylphosphonates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters, methylphosphorothioate, phosphorodithioate, p-ethoxy, and combinations thereof.

In some embodiments, the binding partner or probe is a nucleic acid that is a peptide nucleic acid (PNA), a bisPNA clamp, a pseudocomplementary PNA, a locked nucleic acid (LNA), DNA, RNA, or co-nucleic acids of the above such as DNA-LNA co-nucleic acids. In some instances, the nucleic acid target can also be comprised of any of these elements.

PNAs are DNA analogs having their phosphate backbone replaced with 2-aminoethyl glycine residues linked to nucleotide bases through glycine amino nitrogen and methylenecarbonyl linkers. PNAs can bind to both DNA and RNA targets by Watson-Crick base pairing, and in so doing form stronger hybrids than would be possible with DNA or RNA based probes.

PNAs are synthesized from monomers connected by a peptide bond (Nielsen, P.E. et al. Peptide Nucleic Acids, Protocols and Applications, Norfolk: Horizon Scientific Press, p. 1-19 (1999)). They can be built with standard solid phase peptide synthesis technology. PNA chemistry and synthesis allows for inclusion of amino acids and polypeptide sequences in the PNA design. For example, lysine residues can be used to introduce positive charges in the PNA backbone. All chemical approaches available for the modifications of amino acid side chains are directly applicable to PNAs.

PNA has a charge-neutral backbone, and this attribute leads to fast hybridization rates of PNA to DNA (Nielsen, P.E. et al. Peptide Nucleic Acids, Protocols and

Applications, Norfolk: Horizon Scientific Press, p. 1-19 (1999)). The hybridization rate can be further increased by introducing positive charges in the PNA structure, such as in the PNA backbone or by addition of amino acids with positively charged side chains (e.g., lysines). PNA can form a stable hybrid with a DNA molecule. The stability of such a hybrid is essentially independent of the ionic strength of its environment (Orum, H. et al., *BioTechniques* 19(3):472-480 (1995)), most probably due to the uncharged nature of PNAs. This provides PNAs with the versatility of being used in vivo or in vitro. However, the rate of hybridization of PNAs that include positive charges is dependent on ionic strength, and thus is lower in the presence of salt.

Several types of PNA designs exist, and these include single strand PNA (ssPNA), bisPNA and pseudocomplementary PNA (pcPNA).

The structure of PNA/DNA complex depends on the particular PNA and its sequence. Single stranded PNA (ssPNA) binds to single stranded DNA (ssDNA) preferably in antiparallel orientation (i.e., with the N-terminus of the ssPNA aligned with the 3' terminus of the ssDNA) and with a Watson-Crick pairing. PNA also can bind to DNA with a Hoogsteen base pairing, and thereby forms triplexes with double stranded DNA (dsDNA) (Wittung, P. et al., *Biochemistry* 36:7973 (1997)).

Single strand PNA is the simplest of the PNA molecules. This PNA form interacts with nucleic acids to form a hybrid duplex via Watson-Crick base pairing. The duplex has different spatial structure and higher stability than dsDNA (Nielsen, P.E. et al. Peptide Nucleic Acids, Protocols and Applications, Norfolk: Horizon Scientific Press, p. 1-19 (1999)). However, when different concentration ratios are used and/or in presence of complimentary DNA strand, PNA/DNA/PNA or PNA/DNA/DNA triplexes can also be formed (Wittung, P. et al., *Biochemistry* 36:7973 (1997)). The formation of duplexes or triplexes additionally depends upon the sequence of the PNA. Thymine-rich homopyrimidine ssPNA forms PNA/DNA/PNA triplexes with dsDNA targets where one PNA strand is involved in Watson-Crick antiparallel pairing and the other is involved in parallel Hoogsteen pairing. Cytosine-rich homopyrimidine ssPNA preferably binds through Hoogsteen pairing to dsDNA forming a PNA/DNA/DNA triplex. If the ssPNA sequence is mixed, it invades the dsDNA target, displaces the DNA strand, and forms a Watson-Crick duplex. Polypurine ssPNA also forms triplex PNA/DNA/PNA with reversed Hoogsteen pairing.

BisPNA includes two strands connected with a flexible linker. One strand is designed to hybridize with DNA by a classic Watson-Crick pairing, and the second is designed to hybridize with a Hoogsteen pairing. The target sequence can be short (e.g., 8 bp), but the bisPNA/DNA complex is still stable as it forms a hybrid with twice as many (e.g., a 16 bp) base pairings overall. The bisPNA structure further increases specificity of their binding. As an example, binding to an 8 bp site with a probe having a single base mismatch results in a total of 14 bp rather than 16 bp.

BisPNAs have multiple modes of binding to nucleic acids (Hansen, G.I. et al., *J. Mol. Biol.* 307(1):67-74 (2001)). One isomer includes two bisPNA molecules instead of one. It is formed at higher bisPNA concentration and has a tendency to rearrange into the complex with a single bisPNA molecule. Other isomers differ in positioning of the linker around the target DNA strands. All the identified isomers still bind to the same binding site/target.

Pseudocomplementary PNA (pcPNA) (Izvolsky, K.I. et al., *Biochemistry* 10908-10913 (2000)) involves two single stranded PNAs added to dsDNA. One pcPNA strand is complementary to the target sequence, while the other is complementary to the displaced DNA strand. As the PNA/DNA duplex is more stable, the displaced DNA generally does not restore the dsDNA structure. The PNA/PNA duplex is more stable than the DNA/PNA duplex and the PNA components are self-complementary because they are designed against complementary DNA sequences. Hence, the added PNAs would rather hybridize to each other. To prevent the self-hybridization of pcPNA units, modified bases are used for their synthesis including 2,6-diamiopurine (D) instead of adenine and 2-thiouracil (^{S}U) instead of thymine. While D and ^{S}U are still capable of hybridization with T and A respectively, their self-hybridization is sterically prohibited.

Locked nucleic acid (LNA) molecules form hybrids with DNA, which are at least as stable as PNA/DNA hybrids (Braasch, D.A. et al., *Chem & Biol.* 8(1):1-7(2001)). Therefore, LNA can be used just as PNA molecules would be. LNA binding efficiency can be increased in some embodiments by adding positive charges to it. LNAs have been reported to have increased binding affinity inherently.

Commercial nucleic acid synthesizers and standard phosphoramidite chemistry are used to make LNAs. Therefore, production of mixed LNA/DNA sequences is as simple as that of mixed PNA/peptide sequences. The stabilization effect of LNA monomers is not an additive effect. The monomer influences conformation of sugar

rings of neighboring deoxynucleotides shifting them to more stable configurations (Nielsen, P.E. et al. Peptide Nucleic Acids, Protocols and Applications, Norfolk: Horizon Scientific Press, p. 1-19 (1999)). Also, lesser number of LNA residues in the sequence dramatically improves accuracy of the synthesis. Naturally, most biochemical approaches for nucleic acid conjugations are applicable to LNA/DNA constructs.

Other backbone modifications, particularly those relating to PNAs, include peptide and amino acid variations and modifications. Thus, the backbone constituents of PNAs may be peptide linkages, or alternatively, they may be non-peptide linkages. Examples include acetyl caps, amino spacers such as 8-amino-3,6-dioxaoctanoic acid (referred to herein as O-linkers), amino acids such as lysine (particularly useful if positive charges are desired in the PNA), and the like. Various PNA modifications are known and probes incorporating such modifications are commercially available from sources such as Boston Probes, Inc.

In some instance, for example those embracing nucleic acid analytes, sequence-specific probes may be used. "Sequence-specific" when used in the context of a probe for a nucleic acid means that the probe recognizes a particular linear (or quasi-linear) arrangement of nucleotides or derivatives thereof. In preferred embodiments, the probe is itself composed of nucleic acid elements such as DNA, RNA, PNA and LNA elements and combinations thereof (as discussed below). In preferred embodiments, the linear arrangement includes contiguous nucleotides or derivatives thereof that each binds to a corresponding complementary nucleotide in the probe. In some embodiments, however, the sequence may not be contiguous as there may be one, two, or more nucleotides that do not have corresponding complementary residues on the probe.

Any molecule that is capable of recognizing a polymer such as a nucleic acid with structural or sequence specificity can be used as a sequence-specific probe. In most instances, such probes will form at least a Watson-Crick bond with a nucleic acid analyte. In other instances, the nucleic acid probe can form a Hoogsteen bond with the nucleic acid analyte, thereby forming a triplex. A nucleic acid probe that binds by Hoogsteen binding enters the major groove of a nucleic acid analyte and hybridizes with the bases located there. Examples of these latter probes include molecules that recognize and bind to the minor and major grooves of nucleic acids (e.g., some forms of antibiotics). In some embodiments, the nucleic acid probes can form both Watson-Crick

and Hoogsteen bonds with the nucleic acid polymer. BisPNA probes, for instance, are capable of both Watson-Crick and Hoogsteen binding to a nucleic acid.

The nucleic acid probes of the invention can be any length ranging from at least 4 nucleotides to in excess of 1000 nucleotides. In preferred embodiments, the probes are 5-100 nucleotides in length, more preferably between 5-25 nucleotides in length, and even more preferably 5-12 nucleotides in length. The length of the probe can be any length of nucleotides between and including the ranges listed herein, as if each and every length was explicitly recited herein. Thus, the length may be at least 5 nucleotides, at least 10 nucleotides, at least 15 nucleotides, at least 20 nucleotides, or at least 25 nucleotides, or more, in length. It should be understood that not all residues of the probe need hybridize to complementary residues in the nucleic acid target. For example, the probe may be 50 residues in length, yet only 25 of those residues hybridize to the nucleic acid target. Preferably, the residues that hybridize are contiguous with each other.

The probes are preferably single stranded, but they are not so limited. For example, when the probe is a bisPNA it can adopt a secondary structure with the nucleic acid analyte resulting in a triple helix conformation, with one region of the bisPNA clamp forming Hoogsteen bonds with the backbone of the polymer and another region of the bisPNA clamp forming Watson-Crick bonds with the nucleotide bases of the polymer.

The nucleic acid probe hybridizes to a complementary sequence within the nucleic acid analyte. The specificity of binding can be manipulated based on the hybridization conditions. For example, salt concentration and temperature can be modulated in order to vary the range of sequences recognized by the nucleic acid probes. Those of ordinary skill in the art will be able to determine optimum conditions for a desired specificity.

Times and conditions sufficient to allow specific binding of analytes to their respective binding partners will depend upon the nature of such analytes and binding partners but will generally be known or ascertainable using routine experimentation by one of ordinary skill in the art or by reference to the prior art. (See, for example, Maniatis' Handbook of Molecular Biology.)

Probes may be labeled, for example, using a detectable label. A detectable label is a moiety, the presence of which can be ascertained directly or indirectly. Generally, detection of the label involves the creation of a detectable signal such as for example an

emission of energy. The label may be of a chemical, peptide or nucleic acid nature although it is not so limited. The nature of label used will depend on a variety of factors, including the nature of the analysis being conducted, the type of the energy source and detector used and the type of polymer, analyte, probe and primary and secondary analyte-specific binding partners. The label should be sterically and chemically compatible with the constituents to which it is bound.

The label can be detected directly for example by its ability to emit and/or absorb electromagnetic radiation of a particular wavelength. A label can be detected indirectly for example by its ability to bind, recruit and, in some cases, cleave another moiety which itself may emit or absorb light of a particular wavelength (e.g., an epitope tag such as the FLAG epitope, etc.). Generally the detectable label can be selected from the group consisting of directly detectable labels such as a fluorescent molecule (e.g., fluorescein, rhodamine, tetramethylrhodamine, R-phycoerythrin, Cy-3, Cy-5, Cy-7, Texas Red, Phar-Red, allophycocyanin (APC), fluorescein amine, eosin, dansyl, umbelliferone, 5-carboxyfluorescein (FAM), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), 6 carboxyrhodamine (R6G), N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), 6-carboxy-X-rhodamine (ROX), 4-(4'-dimethylaminophenylazo) benzoic acid (DABCYL), 5-(2'-aminoethyl) aminonaphthalene-1-sulfonic acid (EDANS), 4-acetamido-4'-isothiocyanatostilbene-2, 2'disulfonic acid, acridine, acridine isothiocyanate, r-amino-N-(3-vinylsulfonyl)phenylnaphthalimide-3,5, disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)maleimide, anthranilamide, Brilliant Yellow, coumarin, 7-amino-4-methylcoumarin, 7-amino-4-trifluoromethylcouluarin (Coumarin 151), cyanosine, 4', 6-diaminidino-2-phenylindole (DAPI), 5', 5"-diaminidino-2-phenylindole (DAPI), 5', 5"-dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red), 7-diethylamino-3- (4'-isothiocyanatophenyl) -4-methylcoumarin diethylenetriamine pentaacetate, 4,4'-diisothiocyanatodihydro-stilbene-2, 2'-disulfonic acid, 4,4'-diisothiocyanatostilbene-2, 2'-disulfonic acid, 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC), eosin isothiocyanate, erythrosin B, erythrosin isothiocyanate, ethidium, 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF), QFITC (XRITC), fluorescamine, IR144, IR1446, Malachite Green isothiocyanate, 4-methylumbelliferone, ortho cresolphthalein, nitrotyrosine, pararosaniline, Phenol Red, B-phycoerythrin, o-phthaldialdehyde, pyrene, pyrene butyrate, succinimidyl 1-pyrene butyrate, Reactive Red 4 (Cibacron™, Brilliant Red 3B-A), lissamine rhodamine B sulfonyl chloride, rhodamine B, rhodamine 123,

rhodamine X, sulforhodamine B, sulforhodamine 101, sulfonyl chloride derivative of sulforhodamine 101, tetramethyl rhodamine, riboflavin, rosolic acid, and terbium chelate derivatives), a chemiluminescent molecule, a bioluminescent molecule, a quantum dot (described for example in U.S. Patent No. 6,207,392 and commercially available from Quantum Dot Corporation and Evident Technologies), a gold nanocrystal, and the like. Other detectable labels can also be used with different detector devices. These include a chromogenic molecule, a radioisotope (e.g., P^{32} or H^3, ^{14}C, ^{125}I and ^{131}I), an electron spin resonance molecule (such as for example nitroxyl radicals), an optical or electron density molecule, an electrical charge transducing or transferring molecule, an electromagnetic molecule such as a magnetic or paramagnetic bead or particle, a semiconductor nanocrystal or nanoparticle, a nuclear magnetic resonance molecule, and the like.

The detectable label can also be selected from the group consisting of indirectly detectable labels such as an affinity molecule, a ligand, a receptor, a biotin molecule, an avidin molecule, a streptavidin molecule, an antigen (e.g., epitope tags such as the FLAG or HA epitope), a hapten (e.g., biotin, pyridoxal, digoxigenin fluorescein and dinitrophenol), an antibody, an antibody fragment, and the like. Antibody fragments include Fab, $F(ab)_2$, Fd and antibody fragments which include a CDR3 region.

Secondary analyte binding partners such as nucleic acid probes can be synthesized in a manner that incorporates fluorophores directly into the growing nucleic acid. For example, this latter labeling can be accomplished by chemical means or by the introduction of active amino or thiol groups into nucleic acids. (Proudnikov and Mirabekov, Nucleic Acid Research, 24:4535-4532, 1996.) An extensive description of modification procedures that can be performed on a nucleic acid polymer can be found in Hermanson, G.T., Bioconjugate Techniques, Academic Press, Inc., San Diego, 1996, which is incorporated by reference herein. One of the methods is based on the introduction of aldehyde groups by partial depurination of DNA. Fluorescent labels with an attached hydrazine group are efficiently coupled with the aldehyde groups and the hydrazine bonds are stabilized by reduction with sodium labeling efficiencies around 60%. The reaction of cytosine with bisulfite in the presence of an excess of an amine fluorophore leads to transamination at the N4 position (Hermanson, 1996). Reaction conditions such as pH, amine fluorophore concentration, and incubation time and temperature affect the yield of products formed. At high concentrations of the amine fluorophore (3M), transamination can approach 100% (Draper and Gold, 1980).

In addition to the above method, it is also possible to synthesize nucleic acids de novo (e.g., using automated nucleic acid synthesizers) using fluorescently labeled nucleotides. Such nucleotides are commercially available from suppliers such as Amersham Pharmacia Biotech, Molecular Probes, and New England Nuclear/Perkin Elmer.

As used herein, "conjugated" means two entities stably bound to one another by any physicochemical means. It is important that the nature of the attachment is such that it does not substantially impair the effectiveness of either entity. Keeping these parameters in mind, any covalent or non-covalent linkage known to those of ordinary skill in the art is contemplated unless explicitly stated otherwise herein. Such means and methods of attachment are known to those of ordinary skill in the art. Conjugation can be performed using standard techniques common to those of ordinary skill in the art. For example, U.S. Patent Nos. 3,940,475 and 3,645,090 demonstrate conjugation of fluorophores and enzymes to antibodies.

The detectable labels can be conjugated to all suitable components of the system by non-covalent means, whether directly or indirectly. Non-covalent conjugation includes hydrophobic interactions, ionic interactions, high affinity interactions such as biotin-avidin and biotin-streptavidin complexation and other affinity interactions. An example is the avidin/streptavidin and biotin binding interaction.

Linkers and/or spacers may be used to conjugate components in some instances.

Optical detectable signals are generated, detected and stored in a database. The signals can be analyzed to determine structural information about the nucleic acid. The signals can be analyzed by assessing the intensity of the signal to determine structural information about the nucleic acid. The computer may be the same computer used to collect data about the nucleic acids, or may be a separate computer dedicated to data analysis. A suitable computer system to implement embodiments of the present invention typically includes an output device which displays information to a user, a main unit connected to the output device and an input device which receives input from a user. The main unit generally includes a processor connected to a memory system via an interconnection mechanism. The input device and output device also are connected to the processor and memory system via the interconnection mechanism. Computer programs for data analysis of the detected signals are readily available from CCD (charge coupled device) manufacturers.

Equivalents

It should be understood that the preceding is merely a detailed description of certain embodiments. It therefore should be apparent to those of ordinary skill in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention, and with no more than routine experimentation.

All references, patents and patent applications that are recited in this application are incorporated by reference herein in their entirety.

Claims

1. A method for detecting an analyte in a sample comprising

contacting a primary analyte binding partner immobilized on an array with a sample for a time and under conditions sufficient to allow an analyte in the sample to bind specifically to the primary analyte binding partner,

contacting an analyte specifically bound to the primary analyte binding partner with a secondary analyte binding partner that is conjugated to a fluorophore, and

detecting fluorophores conjugated to a single secondary analyte binding partner and bound to the array using a non-averaged method,

wherein the fluorophores bound to the array are indicative of analytes in the sample.

2. The method of claim 1, wherein the array is comprised of a plurality of different primary analyte binding partners each plurality immobilized on a known region of the array.

3. The method of claim 2, wherein the plurality is at least 100 different primary analyte binding partners.

4. The method of claim 2, wherein the secondary analyte binding partner is a plurality of different secondary analyte binding partners.

5. The method of claim 4, wherein the plurality of different secondary analyte binding partners is at least 100 different secondary analyte binding partners.

6. The method of claim 4, wherein the plurality of secondary analyte binding partners are conjugated to identical fluorophores.

7. The method of claim 1, wherein individual fluorophores are detected using a multi-pixel charge coupled device (CCD) camera.

8. The method of claim 7, wherein the multi-pixel CCD camera has a pixel array of at least 128 x 128 pixels.

9. The method of claim 7, wherein the multi-pixel CCD camera has a magnification range of 1-100.

10. The method of claim 7, wherein multi-pixel CCD camera has an optical resolution of one micron.

11. The method of claim 1, further comprising enumerating the fluorophores bound to the array.

12. A chip comprising a configuration comprising

an inlet port,

an inlet channel,

a reaction chamber comprising at least one array of primary analyte binding partners,

an outlet channel, and

an outlet port,

wherein the ports, channels and chamber all comprise and are connected by smooth walls, and wherein the total volume of the channels and chamber is equal to or less than 1 microliter.

13. The chip of claim 12, wherein the ports, channels and chamber are defined by the absence of hydrophobic ink on the chip surface.

14. The chip of claim 12, wherein the chip is made from low fluorescence glass.

15. The chip of claim 12, wherein the chip is coated with metal and metal oxide.

16. The chip of claim 12, wherein the inlet and/or outlet channel has a length of 1-20 mm.

17. The chip of claim 12, wherein the outlet channel has a width that decreases towards the outlet port.

18. The chip of claim 12, wherein the inlet and outlet channels have a combined volume that is less than 1-10% of the reaction chamber volume.

19. The chip of claim 12, further comprising a fiducial.

20. The chip of claim 12, wherein the reaction chamber comprises at least 2 arrays.

21. The chip of claim 12, wherein chip comprises a plurality of configurations.

22. The chip of claim 21, wherein the plurality of configurations are identical to each other.

23. The chip of claim 12, wherein the array is at least a 10 x 10 array.

24. The chip of claim 20, wherein each of the at least 2 arrays is at least a 10 x 10 array.

25. A method for detecting an analyte in a sample comprising

contacting a primary analyte binding partner immobilized on an array with a sample for a time and under conditions sufficient to allow an analyte in the sample to bind specifically to the primary analyte binding partner, wherein analytes in the sample are conjugated to fluorophores, and

detecting fluorophores that are conjugated to a single analyte and bound to the array using a non-averaged method,

wherein the fluorophores bound to the array are indicative of analytes in the sample.

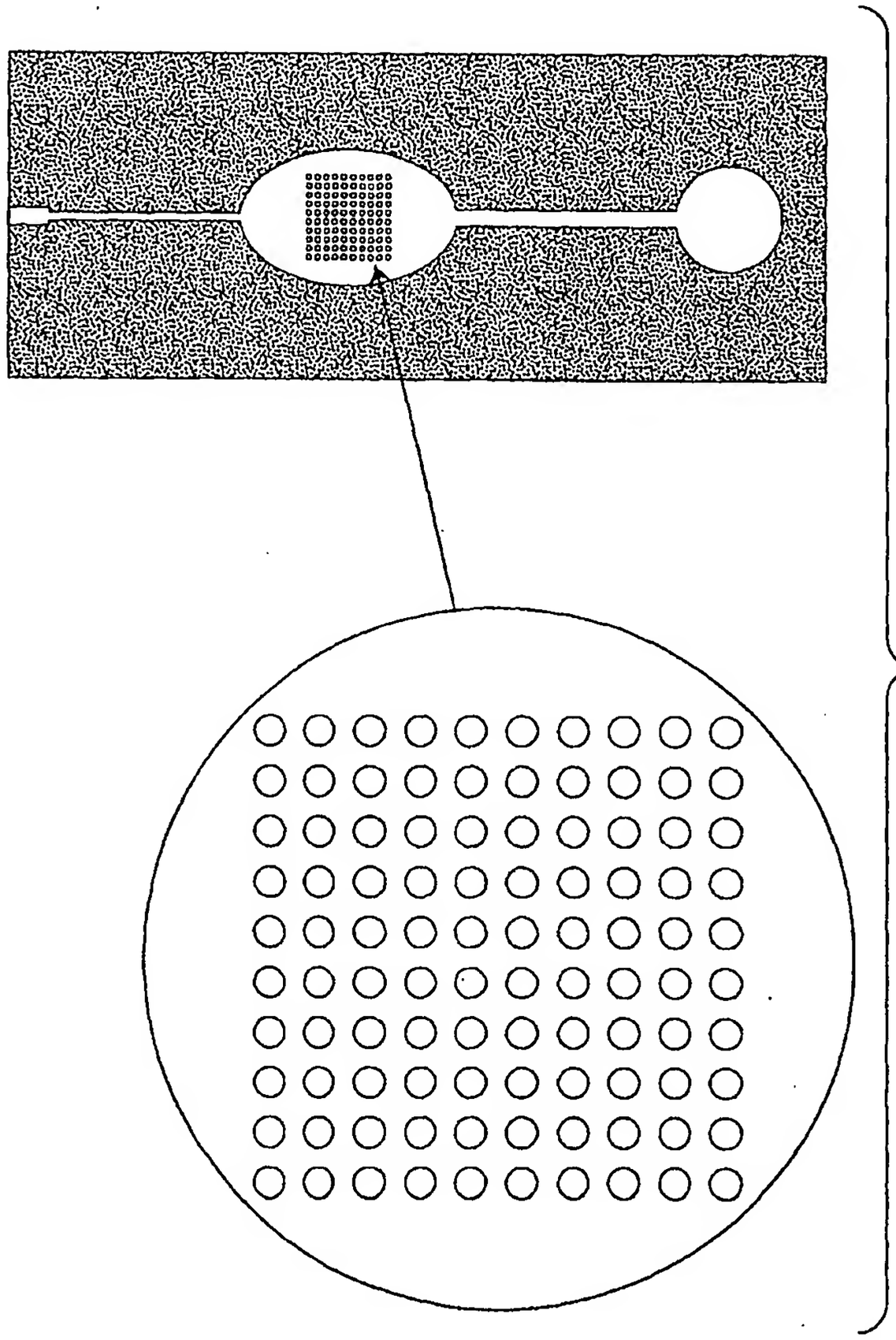
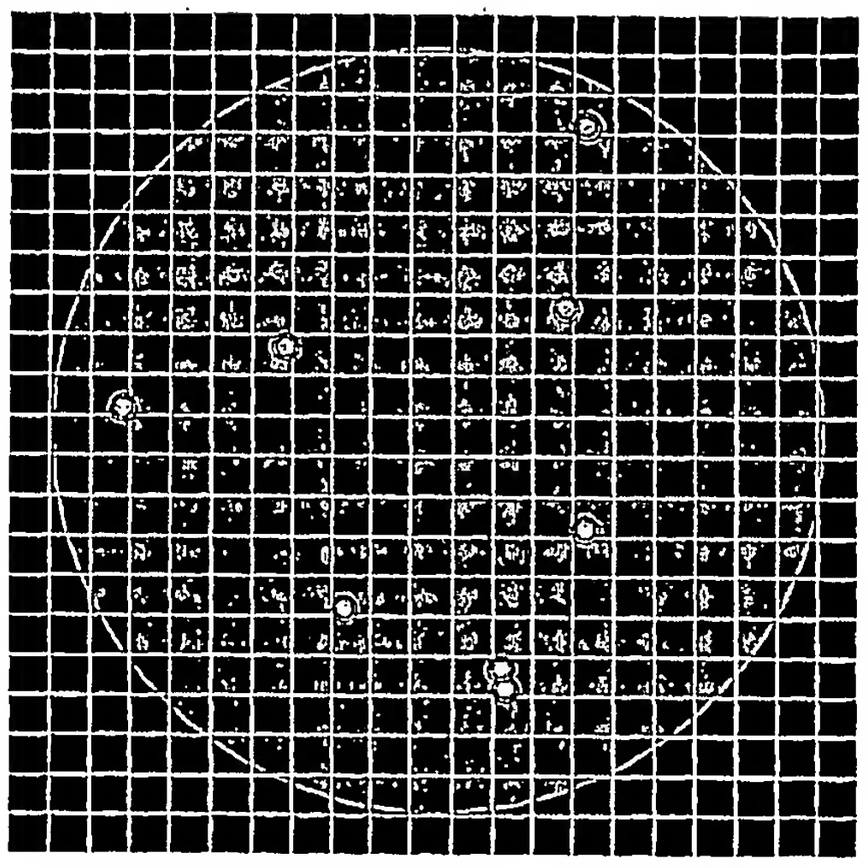


Fig. 1



BACKGROUND FROM ILLUMINATED SPOT

Fig. 2

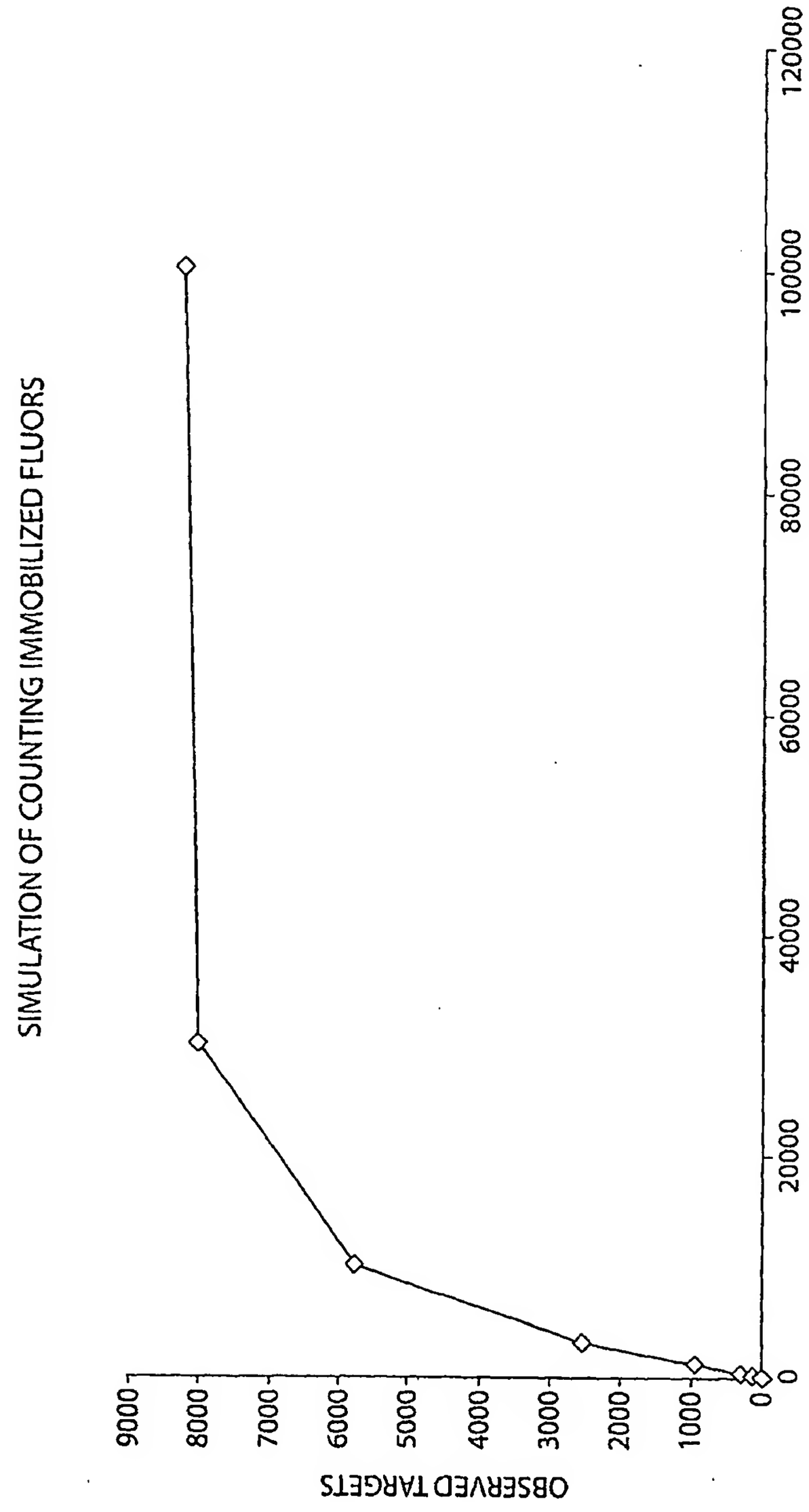
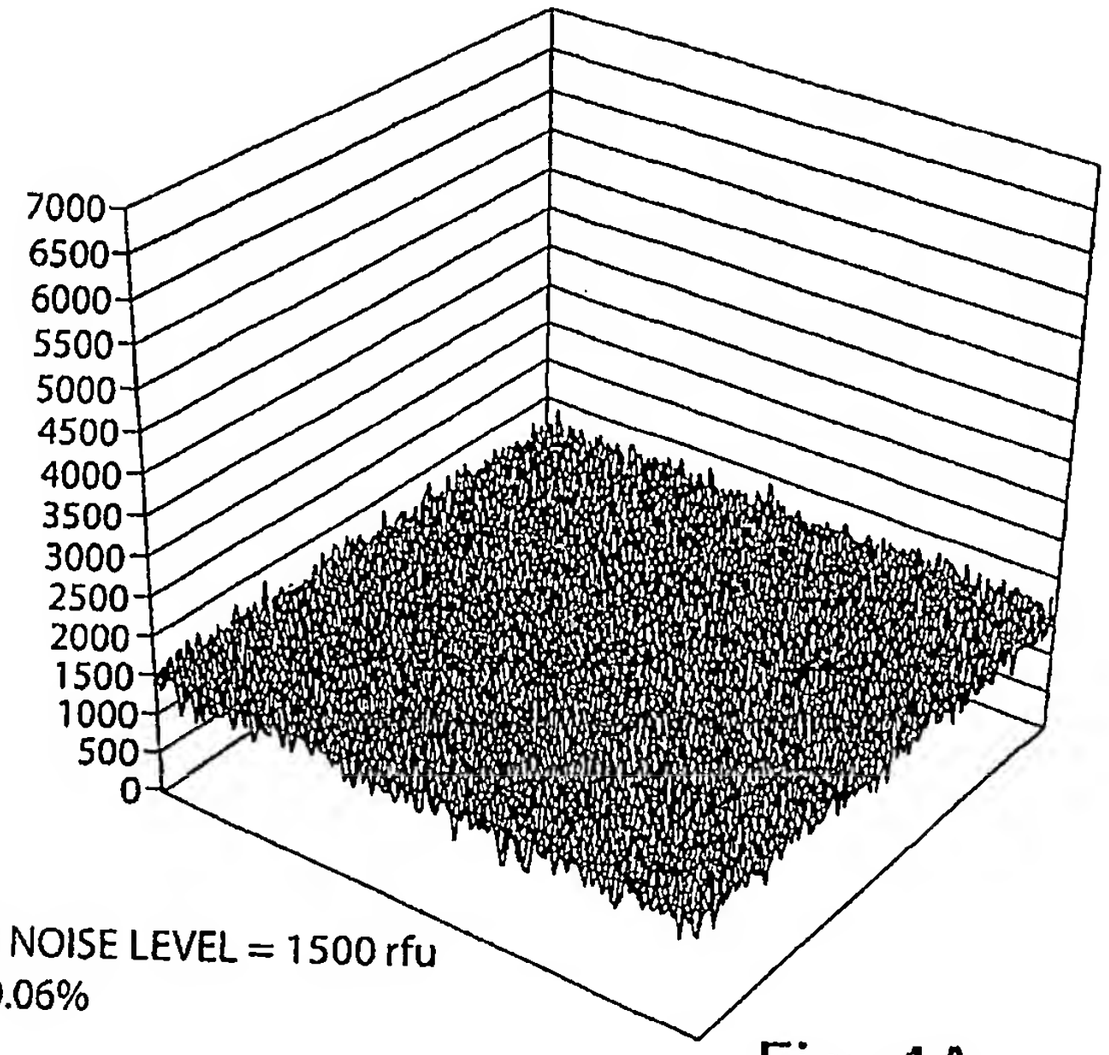
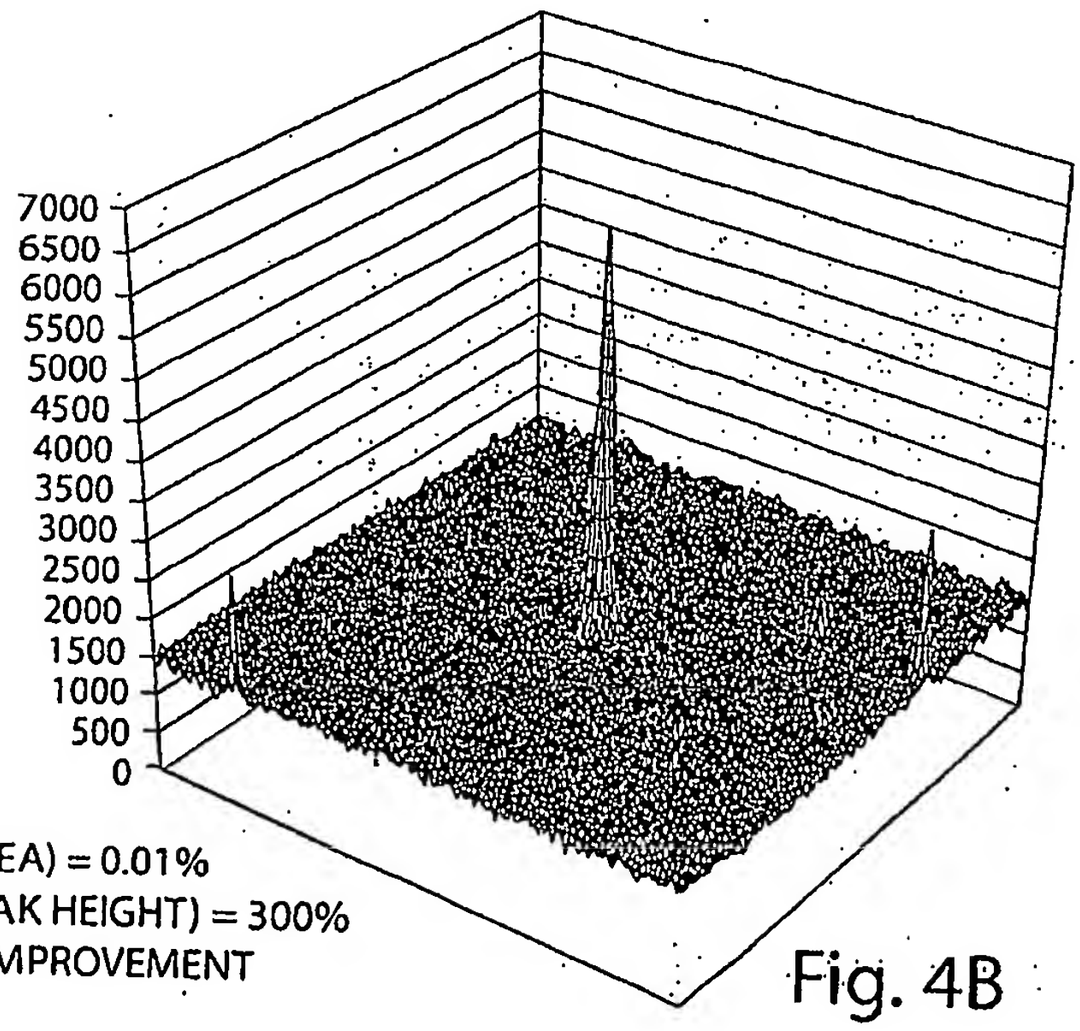

SIMULATION OF COUNTING IMMOBILIZED FLUORS
OBSERVED TARGETS
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
ACTUAL TARGETS
0
20000
40000
60000
80000
100000
120000

FIG. 3



MEAN NOISE LEVEL = 1500 rfu
CV = 0.06%

Fig. 4A



S/N (AREA) = 0.01%
S/N (PEAK HEIGHT) = 300%
4-LOG IMPROVEMENT

Fig. 4B